Clinica Chimica Acta 488 (2019) 40–49

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Colorimetric detection of *PCA3* in urine for prostate cancer diagnosis using thiol-labeled PCR primer and unmodified gold nanoparticles

Khin Phyu Pyar Htoo[a,b], Vichanan Yamkamon[c], Sakda Yainoy[a], Thummaruk Suksrichavalit[d], Wit Viseshsindh[e], Warawan Eiamphungporn[a,*]

[a] Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
[b] Department of Medical Laboratory Technology, University of Medical Technology, Mandalay, Myanmar
[c] Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
[d] Center of Data Mining and Biomedical Informatics, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand
[e] Department of Surgery, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

ARTICLE INFO

Keywords:
Colorimetric assay
Gold nanoparticle
PCA3
Prostate cancer
Urine

ABSTRACT

Background: *PCA3*, a non-coding RNA, has been approved as a potential urinary biomarker for prostate cancer. However, *PCA3* urine tests have some limitations. Therefore, we developed a colorimetric method for *PCA3* detection in urine.

Methods: The assay was based on interactions between unmodified gold nanoparticles (AuNPs) and thiolated PCR products. Thiolated PCR products were amplified by RT-PCR using a thiol-labeled primer at the 5′ end. Thiolated products of *PCA3* bound to the surface of AuNPs and led to the prevention of salt-induced aggregation (red color). In the absence of the PCR products, AuNPs changed their color from red to blue due to the salt-induced aggregation. These changes were detected by the naked eye and spectrophotometer.

Results: Our assay was specific for *PCA3* in prostate cancer cell lines with a visual detection limit of 31.25 ng/reaction. The absorption ratio 520/640 nm was linear against PCR product concentration (R^2 = 0.9798) in the reaction. This method is promising for discrimination of prostate cancer patients from both healthy controls and benign prostatic hyperplasia patients according to their urinary *PCA3* expression levels.

Conclusions: This study established a simple, rapid, sensitive and specific assay for *PCA3* detection which may be applicable for prostate cancer diagnosis.

1. Introduction

Prostate cancer (PCa) is one of the most common types of malignancy worldwide and is the second leading cause of cancer death among men [1,2]. This cancer tends to be asymptomatic and slow growing, often with onset in young men, but usually not detected until the age of 40–50 years [3]. The prevalence of prostate cancer has a strong ethnic propensity, with a higher incidence among Europeans and African Americans than in others [4]. The conventional methods for PCa screening recommended by the American Cancer Society are serum prostate specific antigen (PSA) testing and digital rectal examination (DRE) [5]. However, these methods have some drawbacks due to their sensitivity, specificity and accuracy [6–8]. In 2018, the U.S. Preventive Services Task Force (USPSTF) issued new guidelines stating that the PSA test may provide little benefit for some men and that it should not be used for routine screening. Rather, it should be offered for selected patients depending on individual circumstances [9]. Therefore, the development of specific prostate cancer markers, and methods for detecting these markers, remains crucial.

In 1999, Prostate Cancer Antigen gene 3 (*PCA3*) was determined to be a gene specifically expressed in PCa [10]. The *PCA3* gene is located on the long arm of chromosome 9, is 23 kb long, and contains four exons. According to various termination codons, *PCA3* RNA is not translated into protein and is defined as a long non-coding RNA [10]. The gene is overexpressed in cancer tissue from 10- to 100-fold in comparison with the expression in adjacent benign prostate tissue.

Abbreviations: AuNP, gold nanoparticle; RT-PCR, reverse transcriptase polymerase chain reaction; *PCA3*, prostate cancer antigen 3; PCa, prostate cancer; DRE, digital rectal examination; PSA, prostate specific antigen; BPH, benign prostate hyperplasia; FDA, US Food and Drug Administration; USPSTF, US Preventive Services Task Force; ATCC, American Type Culture Collection; SPR, surface plasmon resonance; NTC, no-target control; PBS, phosphate buffer saline; FBS, fetal bovine serum; EGF, epidermal growth factor; BPE, bovine pituitary extract

* Corresponding author.
E-mail address: warawan.eia@mahidol.ac.th (W. Eiamphungporn).

https://doi.org/10.1016/j.cca.2018.10.036
Received 6 September 2018; Received in revised form 19 October 2018; Accepted 29 October 2018
Available online 30 October 2018
0009-8981/

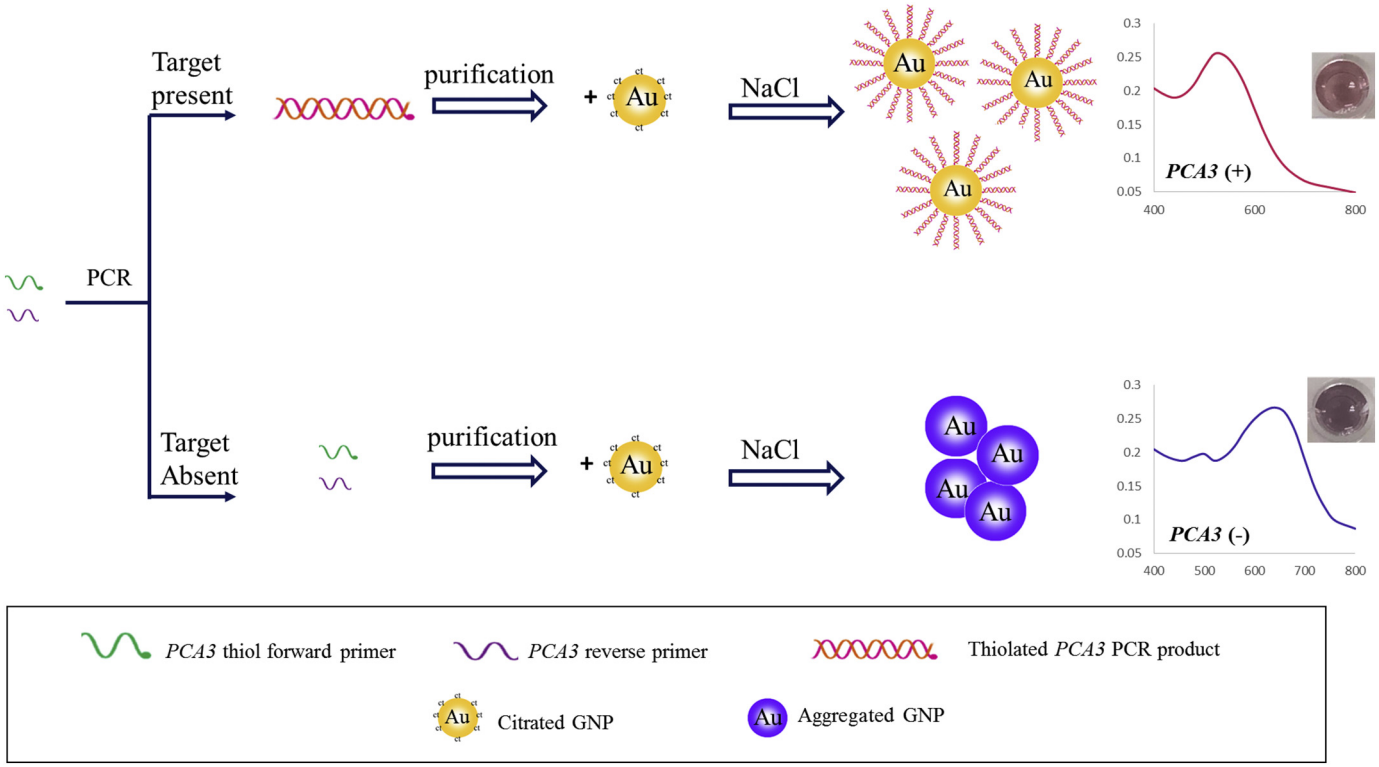

Fig. 1. A schematic representation of the AuNP-based colorimetric assay for detection of *PCA3* in urine sediments.

Table 1
List of primers used in this study.

Primer	Sequence (5′→3′)
PCA3 forward primer (RT-PCR)	GCTGACTTTACCATCTGAGG
PCA3 thiolated forward primer (RT-PCR)	Thiol-GCTGACTTTACCATCTGAGG
PCA3 reverse primer (RT-PCR)	ATGGAGAATGTTGGGCCCAGAAG
PCA3 forward primer (RT-qPCR)	GCTGACTTTACCATCTGAGG
PCA3 reverse primer (RT-qPCR)	GATCTCTGTGCTTCCTTTTG
GAPDH forward primer [34]	CAGCCTCAAGATCATCAGCA
GAPDH reverse primer [34]	TGTGGTCATGAGTCCTTCCA

Moreover, *PCA3* RNA is also detectable in prostate cancer cells found in urine sediments collected after DRE [11]. For this reason, the *PCA3* gene has shown promise as a non-invasive PCa biomarker. Recently, the Progensa *PCA3* test using the *PCA3/PSA* RNA ratio (PCA3 score) in urine has been developed commercially and approved by the US Food and Drug Administration (FDA) as a diagnostic test for clinical use [12,13]. This test is the first FDA-approved, urine-based molecular diagnostic test recommended for men with elevated serum PSA and a previous negative biopsy [14,15]. When used in combination with other clinical parameters, the use of this test can reduce the need for unnecessary prostate biopsies [16]. The Progensa assay is based on two quantitative nucleic acid amplifications with high sensitivity and specificity [17]. However, it is time-consuming, expensive and requires a sophisticated instrument. Therefore, a simple, rapid and cost-effective method is needed for *PCA3* detection.

Colorimetric assays are simple methods since they enable rapid visual detection without the need for complicated equipment [18–20]. In recent years, metal nanoparticles [particularly gold nanoparticles (AuNPs)] have been extensively employed for colorimetric detection due to their unique optical properties. These include a color change associated with the transition of colloidal AuNPs from dispersed to aggregated states [21,22]. The AuNP aggregation results in a shift in surface plasmon resonance (SPR)-related absorption (~520 nm to ~650 nm) causing a colorimetric change in the test solution from red to blue [23,24]. AuNP-based DNA assays are typically classified as being either 'labeled' or 'label-free' [25]. Labeled AuNPs are customarily modified with single-stranded DNA and allowed to hybridize with a complementary target DNA sequence [26]. This modified-AuNP strategy is complicated, laborious, expensive and time-consuming, therefore it has not been adopted for routine use [27]. To overcome these limitations, unmodified AuNPs are utilized directly for label-free colorimetric detection methods. Unmodified AuNPs provide simple, inexpensive and rapid detection of various DNA sequences [28,29] and RNA sequences [30]. They are usually employed for the detection of amplified PCR products. One strategy involves the use of a thiolated PCR primer combined with unmodified AuNPs [31,32]. Typically, AuNPs will aggregate (blue color) in a salt solution. In the presence of thiol-labeled PCR products, DNA molecules strongly bind to the surface of AuNPs and the long chains of DNA which have abundant negative charges enhance the electrostatic and steric repulsion among AuNPs, which consequently leads to the prevention of the salt-induced aggregation (red color) [29,33]. The color change can be monitored as a shift of SPR absorption or can even be observed by the naked eye. This assay offers further advantages over gel electrophoresis which is laborious and requires the use of ethidium bromide, a known carcinogenic agent, for DNA visualization.

In this study, a simple and rapid colorimetric assay based on unmodified AuNPs and a thiol-labeled PCR primer was developed to detect *PCA3* in urine samples for PCa diagnosis. An overall schematic of this method is presented in Fig. 1. The proposed method provides high sensitivity and specificity. Our method is promising for use with a colorimetric sensor since it is capable of distinguishing the urine of PCa patients from that of both healthy subjects and benign prostatic hyperplasia (BPH) patients.

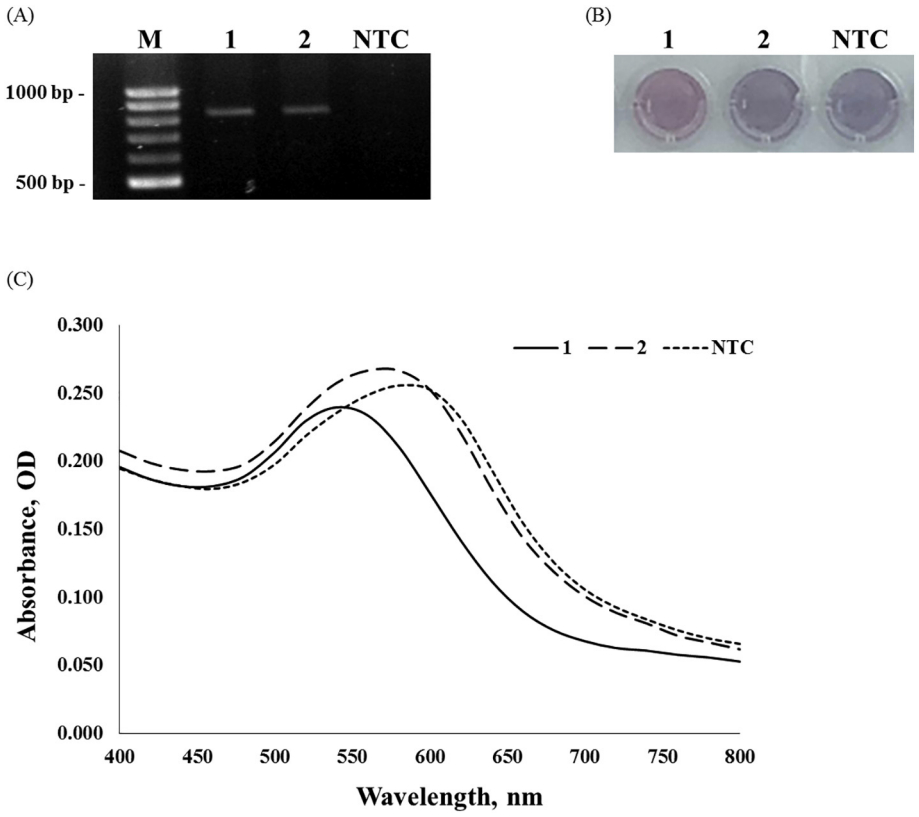

Fig. 2. Effect of thiol-labeled PCR primer on PCR efficiency and salt-induced aggregation of AuNPs. (A) PCR products analyzed by 1.5% agarose gel electrophoresis. Lane M, 100 bp ladder; Lane 1, PCR products from thiol-labeled primer; Lane 2, PCR products from unlabeled primer; NTC, no-target control. (B) Mixtures of AuNPs and PCR products after addition of salt solution. The order of the reactions is similar to gel electrophoresis. (C) Absorption spectra of the AuNP solutions containing the products obtained from thiol-labeled primer, unlabeled primer and NTC.

2. Materials and methods

2.1. Materials and reagents

AuNPs with the size of 10 nm were purchased from Sigma Aldrich (St. Louis, MO, USA). Media and reagents required for cell culture, namely Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM) and sodium pyruvate (100 mM), were obtained from Hyclone (Logan, UT, USA), while keratinocyte serum free medium (K-SFM), fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/mL penicillin, 10 mg/mL streptomycin), bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) were purchased from Gibco (Calsbad, CA, USA). All specific primers and a thiolated forward primer for amplification were ordered from Integrated DNA Technologies, Inc. (Skokie, IL, USA). SsoAdvanced Universal SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). RevertAid First Strand cDNA synthesis kit and Phusion high-fidelity DNA polymerase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). i-Taq plus DNA polymerase was from iNtRON Biotechnology (Gyeonggi-do, South Korea). NucleoSpin gel and PCR clean-up kit was obtained from Macherey-Nagel GmbH & Co. KG (Duren, Germany).

2.2. Cell culture

RWPE-1 (ATCC CRL-11609) prostate epithelial cell line, LNCaP clone FGC (ATCC CRL-1740) and VCaP (ATCC CRL-2876) prostate cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). LNCaP was propagated in RPMI 1640 medium along with 10% FBS and 1% penicillin-streptomycin. VCaP was cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 1 mM sodium pyruvate. For RWPE-1, K-SFM with 5 ng/mL EGF and 50 μg/mL BPE were used for culture. All cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Urine sample preparation

Spot urine samples from 5 healthy male volunteers, first voided post-DRE urine from 5 BPH patients and from 5 PCa patients were provided by the Division of Urology, Department of Surgery, Faculty of Medicine Ramathibodi Hospital, Mahidol University. Diagnosis of patients was made by histopathological analysis after prostate biopsy subsequently. PCa patients were identified with positive biopsy. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (MURA2016/34). Written informed consent was obtained from all subjects in the study. The urine samples were collected and then centrifuged at 4 °C, 3000 rpm for 10 min to separate the cells. Cell pellets were washed twice by PBS, pH 7.0. Finally, TRIzol reagent (Invitrogen, CA, USA) was added to the sediments which were then stored at −20 °C until RNA isolation.

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from the cell pellets of urine as well as from cell lines with TRIzol reagent. 500 μg of total RNA was converted to cDNA using RevertAid First Strand cDNA synthesis kit according to the manufacturer's instructions and stored at −20 °C until use.

2.5. RT-PCR and PCR purification

PCA3 amplification was performed using a thiol-labeled or unlabeled forward primer, and an unlabeled reverse primer. The sequences of primers are shown in Table 1. Thiolated PCR products contained thiol-labeling at the 5′ end. PCR was carried out in a 50 μL reaction mixture containing 0.05 μg of cDNA template, 0.5 μM of each primer, 1× PCR reaction buffer, 0.2 mM dNTPs and 2.5 U i-Taq DNA polymerase. Of note, to amplify *PCA3* from cDNA of clinical samples, 2.5 U Phusion high-fidelity DNA polymerase was applied in the PCR reaction instead of i-Taq DNA polymerase. The cycling procedures were

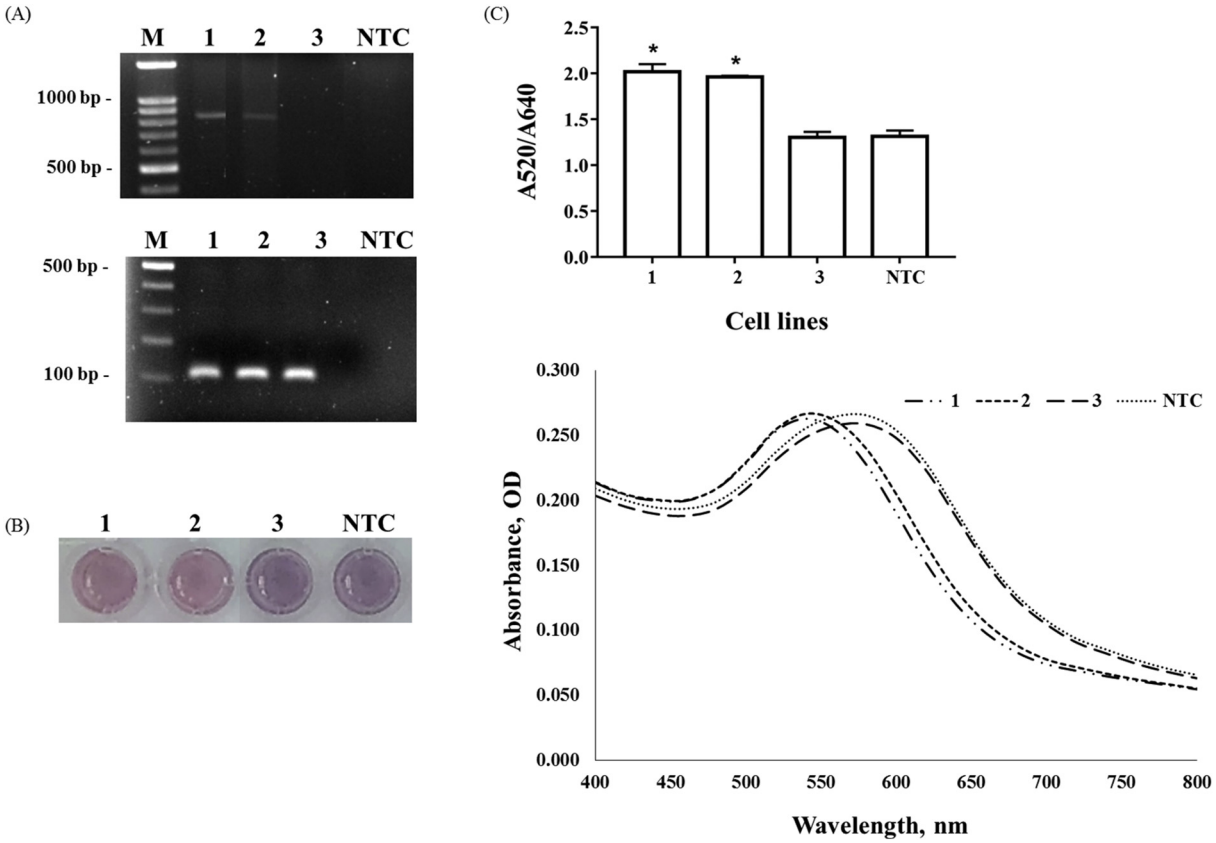

Fig. 3. Specificity analysis of AuNP-based colorimetric assay. (A) PCR products of *PCA3* (upper gel) and *GAPDH* (lower gel) analyzed by 1.5% agarose gel electrophoresis. Amplicon sizes of *PCA3* and *GAPDH* were 838 bp and 106 bp, respectively. Lane M, 100 bp ladder; Lanes 1–3, PCR products from LNCaP, VCaP and RWPE-1, respectively; Lane NTC, no-target control. (B) Visual detection by the naked eye of AuNPs mixed with corresponding thiolated *PCA3* products from LNCaP, VCaP and RWPE-1 in wells 1–3, respectively. (C) UV–vis spectral analysis of the AuNP solutions containing PCR products and NTC. The order of the reactions is similar to gel electrophoresis. A520/A640 ratio determined by UV–vis spectrophotometer. Each bar represents the mean ± SD obtained from three experiments. The statistical analysis utilized the *t*-test. **P*-value < .05 compared with NTC.

set for 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 90 s at 72 °C, and finally extension 10 min at 72 °C. After amplification, 10 μL PCR products were analyzed by agarose-gel electrophoresis. The PCR amplicon size was 838 bp. The rest of PCR products were purified and eluted using gel and PCR clean-up kit according to the manufacturer's instructions. *GAPDH* gene, a housekeeping gene, was used as a control to check cDNA quality. To amplify *GAPDH*, the cycling procedures were performed as followed: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension step of 5 min. The sequences of primers are shown in Table 1. The PCR amplicon size was 106 bp.

2.6. PCA3 detection using AuNP-based colorimetric assay

After PCR purification, 5 μL of thiolated or unlabeled PCR products was added to 80 μL colloidal AuNP solution followed by the addition of 16 μL of 5 M NaCl (0.69 M) to induce aggregation of AuNPs. A no-target control (NTC) was performed as a negative control. All assays were run in triplicate in 96-well microplates. Spectral scanning in the visible region (400–700 nm) was achieved by microplate reader (BioTek Inc., Winooski, USA) after incubation for 5–10 min.

2.7. Specificity and sensitivity tests

The specificity of the assay was determined using 5 μL PCR products amplified from each cell line mixed with the AuNP solution according to the above protocol. To demonstrate the sensitivity of the colorimetric assay, thiolated PCR products of *PCA3* were diluted in a serial two-fold manner (1000 ng to 7.8 ng). Subsequently, 5 μL of each diluted PCR product was assayed in the same manner as previously described.

2.8. qRT-PCR

qRT-PCR was used as a confirmation method. Primers for qRT-PCR are listed in Table 1. Each PCR reaction was composed of 1× SsoAdvanced universal SYBR Green supermix, 0.5 μM of each primer and 25 ng of cDNA template. qRT-PCR for *PCA3* and *GAPDH* was programmed for 3 min at 95 °C, followed by 40 cycles of 20 s at 95 °C and 30 s at 60 °C. The baseline threshold was adjusted and the Ct was analyzed. Melt curve analysis was analyzed by CFX™ manager software 3.1 (Bio-Rad, Hercules, CA, USA). Gel electrophoresis was performed to confirm the presence of PCR products. Finally, the relative expression level of *PCA3* was calculated by a $2^{-\Delta\Delta Ct}$ relative quantification method according to the manufacturer's instruction.

2.9. Statistical analysis

Data were analyzed by SPSS PASW Statistics 25 (SPSS Inc., Chicago, USA) and represented as mean ± standard deviation (SD). The differences between groups in each experiment (samples from cell lines and subjects) were compared using *t*-tests. Statistical significance was defined as a *P*-value < .05.

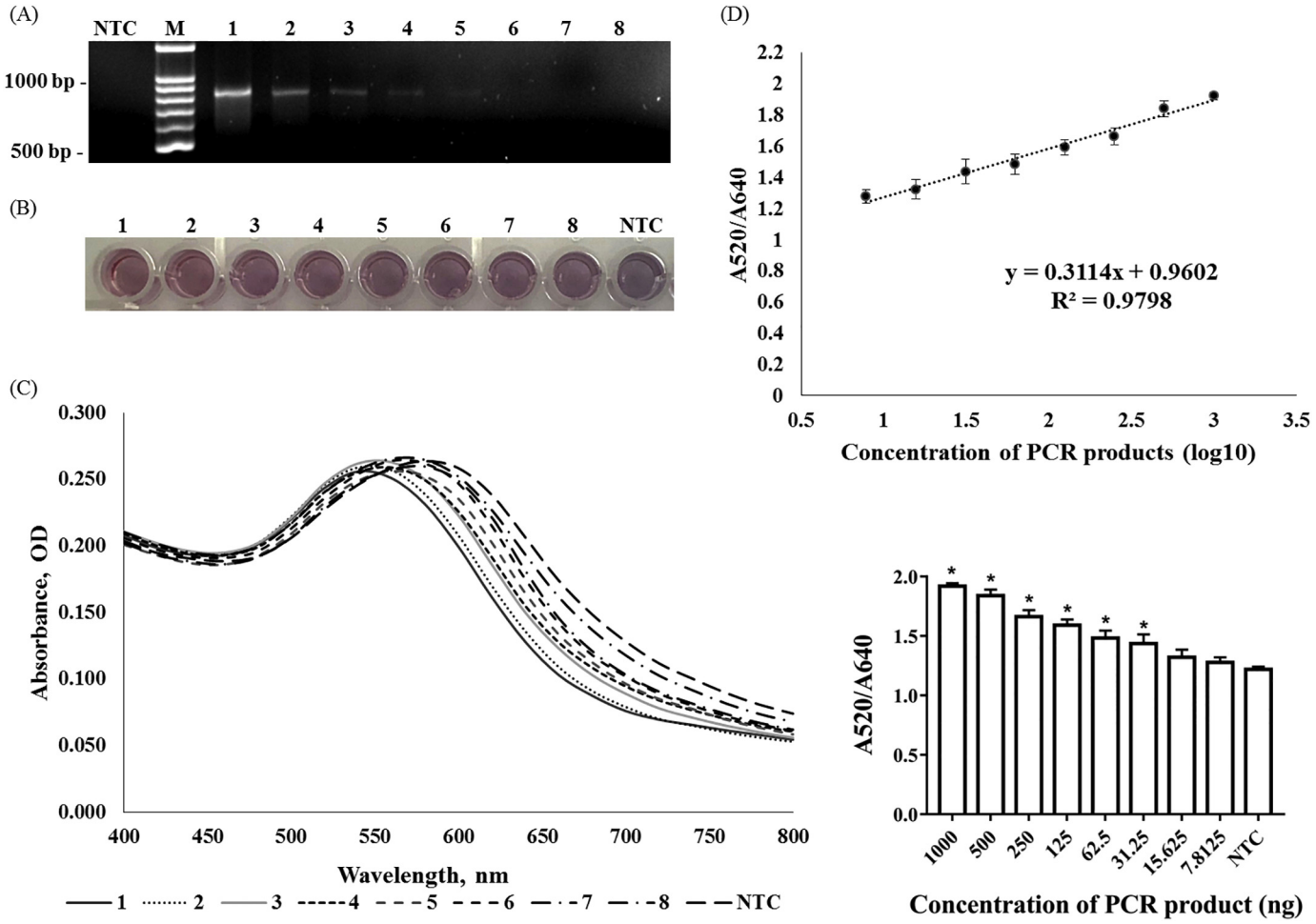

Fig. 4. Sensitivity analysis of AuNP-based colorimetric assay. (A) Agarose gel electrophoresis showing different concentrations of thiolated PCR products of *PCA3*. Lane M, 100 bp ladder; Lanes 1–8, PCR product concentrations as follows: 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 ng, respectively; NTC, no-target control. (B) Mixtures of the corresponding AuNP solutions containing different concentrations of thiolated PCR products after salt adding. The color change was evaluated by the naked eye. (C) Absorption spectra of the AuNP solutions containing the different concentrations of the thiolated PCR products and NTC. The order of the reactions is similar to gel electrophoresis. (D) Calibration curve for different concentrations of PCR products using thiol-labeled primer and the A520/A640 ratios. A520/A640 ratio determined by UV–vis spectrophotometer. Each bar represents the mean ± SD obtained from three experiments. The statistical analysis utilized the *t*-test. **P*-value < .05 compared with NTC.

Table 2
Relative *PCA3* expression level and characteristics of participants.

Sample	Age	Serum PSA (ng/mL)	Final diagnosis	Relative *PCA3* expression level[a]
M1	38	0.168	Normal	ND[b]
M2	34	2.6	Normal	ND
M3	37	1.23	Normal	ND
M4	43	0.148	Normal	ND
M5	35	0.285	Normal	ND
B1	65	9.49	BPH	0.40
B2	79	18.27	BPH	0.07
B3	57	8.0	BPH	0.02
B4	67	7.74	BPH	0.26
B5	51	24.8	BPH	0.06
P1	70	43.03	Prostate cancer	3.73
P2	74	23	Prostate cancer	6.30
P3	71	48.84	Prostate cancer	147.03
P4	55	224	Prostate cancer	142.02
P5	76	38.71	Prostate cancer	20.61
C	63	1.14	BPH	1.0

[a] Comparing to the calibrator (C).
[b] ND = Not detectable.

3. Results

3.1. The effects of thiol-labeled primer on AuNP-based colorimetric assay

In order to evaluate the PCR efficiency, thiol-labeled PCR primer and conventional unlabeled PCR primer were employed for RT-PCR. cDNA from the LNCaP cell line was used as a template. Gel electrophoresis was employed to analyze the PCR products. As shown in Fig. 2A, both primers yielded 838 bp PCR products with similar intensities. This result indicated that the efficiencies of thiol-labeled primer and conventional primer were almost identical. To confirm the role of thiol on AuNPs in preventing their salt-induced aggregation, thiol-labeled and unlabeled PCR products were applied to the AuNP solution. As expected, the AuNP solution with unlabeled PCR products immediately changed from red to blue, while the AuNP solution with thiol-labeled products remained red after adding salt (Fig. 2B). Spectral scanning of red and blue solutions showed peaks at 520 nm and 600 nm, respectively (Fig. 2C). The result suggested that the thiol-labeled primer played a role in blocking the AuNP aggregation induced by salt.

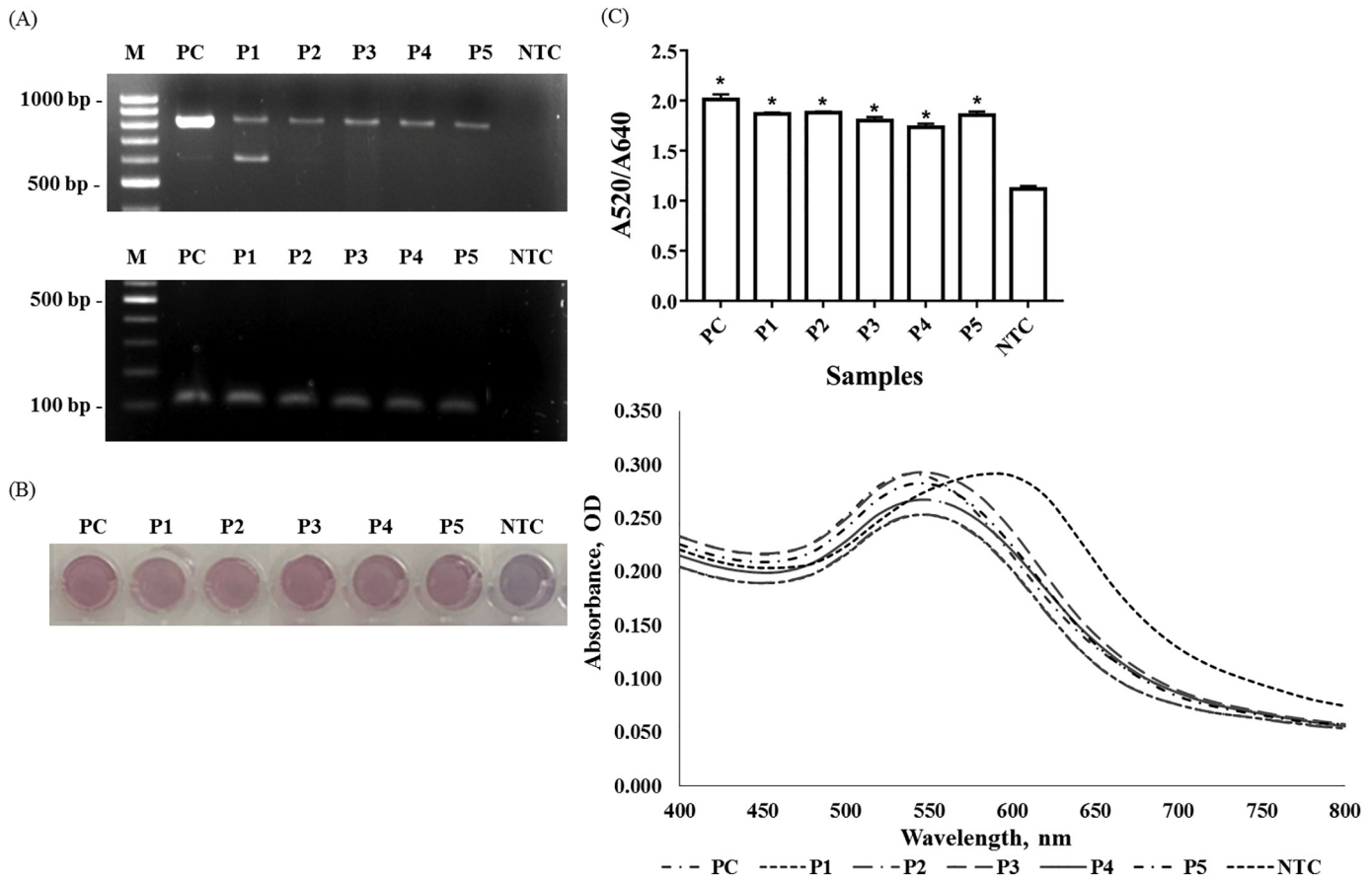

Fig. 5. *PCA3* detection from urine of PCa patients. (A) PCR products of *PCA3* (upper gel) and *GAPDH* (lower gel) analyzed by 1.5% agarose gel electrophoresis. Amplicon sizes of *PCA3* and *GAPDH* were 838 bp and 106 bp, respectively. Lane M, 100 bp ladder; Lane PC, PCR products from LNCaP cells; Lanes P1-P5, PCR products from urine of PCa patients; Lane NTC, no-target control. (B) AuNP-based colorimetric assay. Color change was visualized by the naked eye. The order of the reactions is similar to gel electrophoresis. (C) Absorption spectra of mixtures of AuNP solutions and samples. A520/A650 ratio determined by UV–vis spectrophotometer. Each bar represents the mean ± SD obtained from three experiments. The statistical analysis was evaluated by t-test. **P*-value < .05 compared with NTC.

3.2. Specificity test

The specificity of *PCA3* expression was investigated by using cDNA templates extracted from various cell lines, i.e., LNCaP and VCaP as prostate cancer cell lines and RWPE-1 as a prostate epithelial cell line. The quality of the cDNA templates was examined by RT-PCR using specific primers for the *GAPDH* gene. As shown in Fig. 3A, 106 bp PCR products of *GAPDH* were amplified from the cDNA of all tested cell lines. This result indicated the good quality of the cDNA templates. Notably, 838 bp PCR products of *PCA3* were detected from both LNCaP and VCaP cell lines, while not detected from RWPE-1. In the colorimetric assay, these PCR products were added to AuNP solutions. After adding salt, the AuNP solutions containing thiol-labeled PCR amplicons remained red, whereas color change was observed in the AuNP solutions containing non-amplified PCR products (Fig. 3B). The color could be distinguished by the naked eye. Spectral scanning of each tested solution is shown in Fig. 3C. Moreover, the A520/A640 ratio was used to compare positive (red) and negative (blue) results. The ratios of positive results were significantly greater than those of negative results (Fig. 3C).

3.3. Sensitivity test and detection limit

To verify the sensitivity of this method, different concentrations of PCR products amplified from the LNCaP cell line were applied to the system. Color changes of AuNP solutions were observed by the naked eye and then scanned within the absorption spectra ranging from 400 to 700 nm. The same concentrations of PCR products were also subjected to gel electrophoresis. At < 62.5 ng of PCR products, no band appeared on the gel electrophoresis (Fig. 4A). Interestingly, the red color was obviously detected at 31.25 ng of PCR products. Moreover, the A520/A640 ratio at this concentration was also statistically different than that of the NTC. As a result, the detection limit of the assay was approximately 31.25 ng (Fig. 4B). These results indicated that the proposed colorimetric assay was more sensitive than gel electrophoresis. The absorption spectra were shifted when decrease of PCR product concentrations (Fig. 4C). Notably, the intensity of blue color increased with lower amounts of thiol-labeled amplified DNA. The degree of color change (A520/A640) was linearly dependent on the concentration of thiolated PCR products ($R^2 = 0.9798$) as shown in Fig. 4D. These findings clearly indicated that the presence of thiol-labeled DNA caused an increase in the resistance to salt induced-aggregation of AuNPs.

3.4. PCA3 detection in urine samples using AuNP-based colorimetric assay

After establishing assay specificity and sensitivity, the AuNP-based colorimetric assay was utilized for detection of *PCA3* in clinical samples. Fifteen clinical urinary samples used in this study: 5 were from BPH patients, 5 were from biopsy-proven PCa patients and the remaining 5 were from healthy controls. In order to verify our detection system, all samples were tested for *PCA3* expression by qRT-PCR. The relative *PCA3* expression level in urine from PCa patients was significantly higher than that from both healthy subjects and BPH patients (Table 2). Thiolated PCR products of *PCA3* were amplified from cDNA

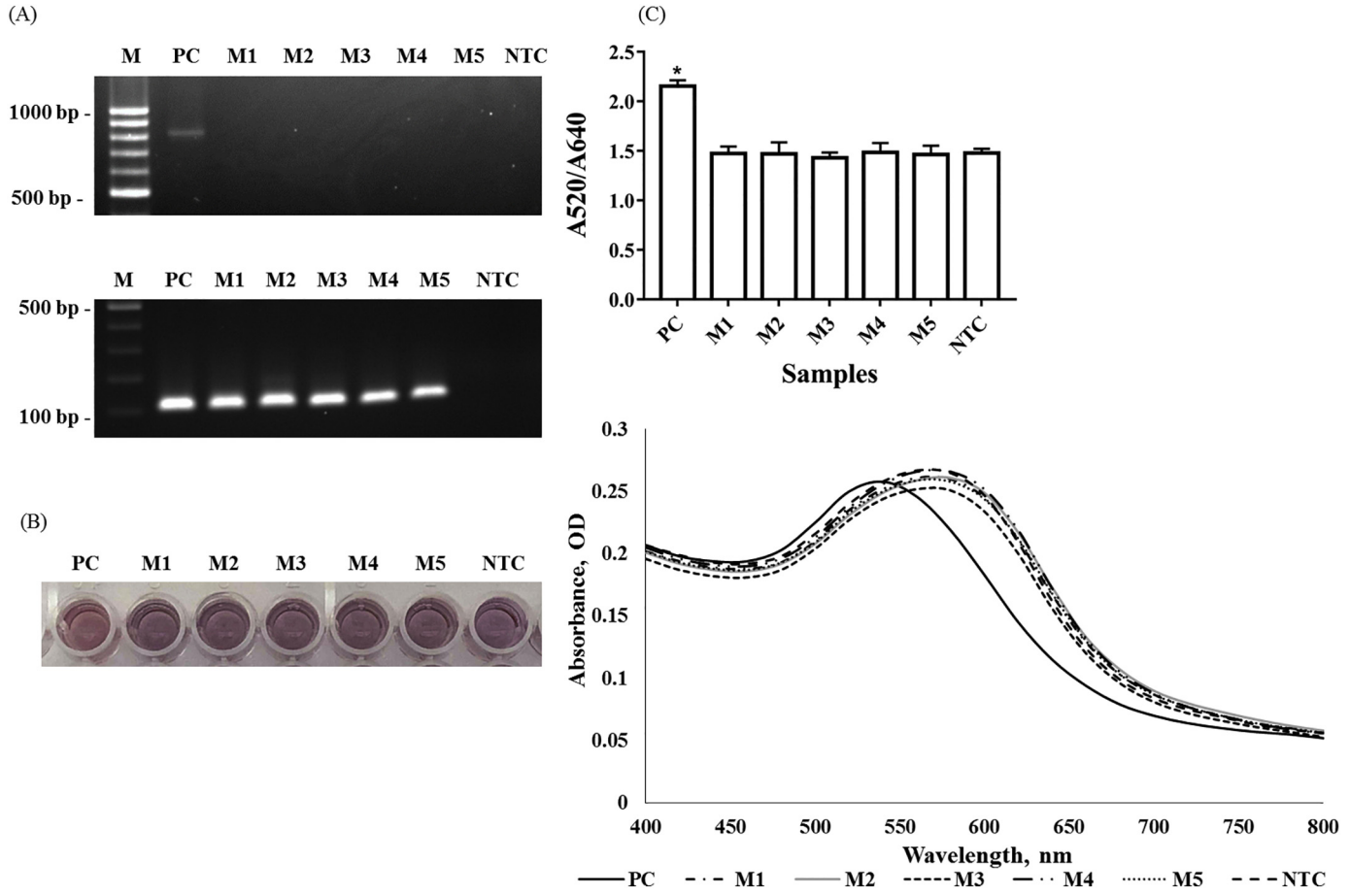

Fig. 6. *PCA3* detection from urine of healthy controls. (A) PCR products of *PCA3* (upper gel) and *GAPDH* (lower gel) analyzed by 1.5% agarose gel electrophoresis. Amplicon sizes of *PCA3* and *GAPDH* were 838 bp and 106 bp, respectively. Lane M, 100 bp ladder; Lane PC, PCR products from LNCaP cells; Lanes M1-M5, PCR products from urine of healthy subjects; Lane NTC, no-target control. (B) AuNP-based colorimetric assay. Color change was visualized by the naked eye. The order of the reactions is similar to gel electrophoresis. (C) Absorption spectra of mixtures of AuNP solutions and samples. A520/A640 ratio determined by UV–vis spectrophotometer. Each bar represents the mean ± SD obtained from three experiments. The statistical analysis was evaluated by t-test. **P*-value < .05 compared with NTC.

of subjects by RT-PCR. The quality of the cDNA samples was investigated by *GAPDH* amplification. By gel electrophoresis, *PCA3* was detected only in PCa samples, while no band was observed in samples from other groups (Figs. 5A, 6A, 7A). Of note, *GAPDH* products were amplified and detected from all samples, reflecting the cDNA integrity (Figs. 5A, 6A, 7A). Thiolated PCR products were added into the AuNP solutions. As expected, no observable color change was detected when thiolated PCR products from PCa patients were tested (Fig. 5B). However, an obvious color change was visualized when samples from both BPH patients and healthy subjects were tested as well as the NTCs (Figs. 6B, 7B). The A520/A640 ratio of each sample was calculated and graphs were plotted as shown in Figs. 5C, 6C, 7C. Notably, the average A520/A640 ratio from all positive samples was significantly greater than that of negative samples (*P*-value < .05) and higher than the cutoff point of 1.5.

4. Discussion

PCA3, a long non-coding RNA, is found to be strongly overexpressed in PCa tissue compared to normal prostate tissue [35]. Moreover, previous studies indicate that the expression of *PCA3* in urine of PCa patients is high, while its expression is low or undetectable in BPH and normal subjects [36–38]. This characteristic of *PCA3* makes it a promising PCa biomarker [39]. Over the past decade, examination of *PCA3* gene expression by various approaches was extensively studied [16,40]. In this process, the Progensa *PCA3* commercial assay (Hologic Gen-Probe, Marlborough, MA, USA) was developed. However, this assay is not ideal for routine clinical screening due to its high cost and need for specialized instrument.

In the present study, the AuNP-based colorimetric assay for determination of *PCA3* in urine was established. This assay used a strategy of combining RT-PCR and AuNP-based colorimetric methods. Unmodified AuNPs and a thiol-labeled primer were used to generate a visual detection readout. To evaluate the PCR efficiency of thiol-labeled and unlabeled primers, PCR reactions were performed using both primers. The results revealed that thiol-labeled primer did not disturb the PCR reaction as compared to the unlabeled primer. This is consistent with the results of previous studies demonstrating that no reduction in band intensity of PCR products when a primer was thiolated [33,41]. Notably, thiol-labeled products prevented salt-induced AuNP aggregation, while neither unlabeled PCR products nor NTC did so (Fig. 2B). Thiolated PCR products bind to unmodified AuNPs as a result of the strong interaction between thiol groups and the gold surface. The DNA grafted on AuNP surfaces forms a thick barrier which protects each particle from coming close to another [42]. These properties cause an increase in steric repulsion between neighboring DNA-bound AuNPs, which results in a resistance to color change due to salt-induced nanoparticle aggregation. On the other hand, AuNPs which lack thiolated PCR products undergo an abrupt and easily observed color change caused by particle aggregation upon the addition of salt solution [30].

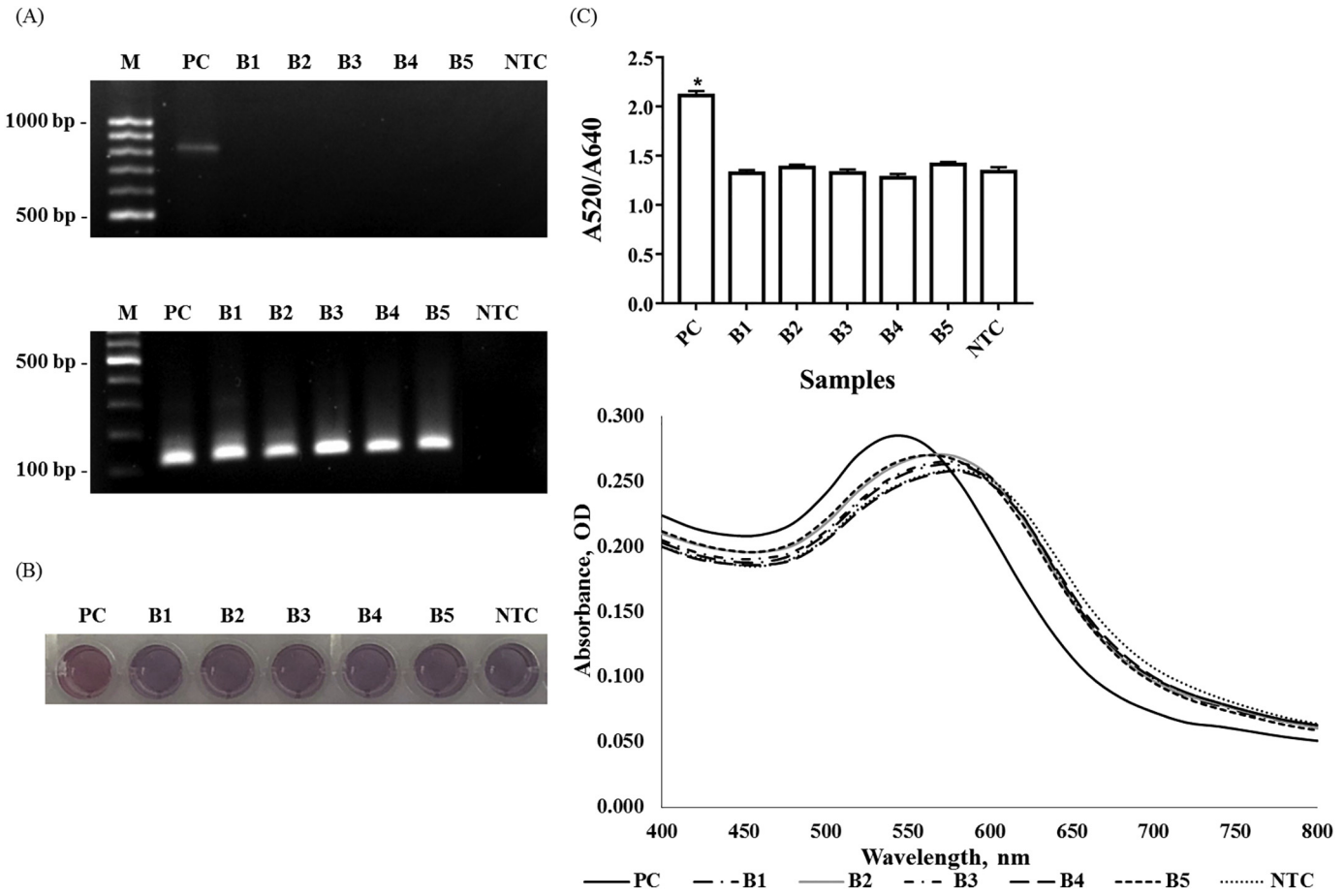

Fig. 7. *PCA3* detection from urine of BPH patients. (A) PCR products of *PCA3* (upper gel) and *GAPDH* (lower gel) analyzed by 1.5% agarose gel electrophoresis. Amplicon sizes of *PCA3* and *GAPDH* were 838 bp and 106 bp, respectively. Lane M, 100 bp ladder; Lane PC, PCR products from LNCaP cells; Lanes B1-B5, PCR products from urine of BPH patients; Lane NTC, no-target control. (B) AuNP-based colorimetric assay. The color change was visualized by the naked eye. The order of the reactions is similar to gel electrophoresis. (C) Absorption spectra of mixtures of AuNP solutions and samples. A520/A640 ratio determined by UV–vis spectrophotometer. Each bar represents the mean ± SD obtained from three experiments. The statistical analysis was evaluated by t-test. **P*-value < .05 compared with NTC.

Our AuNP solutions containing thiolated PCR products remained red after salt addition. The spectral scanning of these solutions displayed an absorption peak at 520 nm representing the dispersed state of the AuNPs. In contrast, a shifted peak (approximately 600 nm) was observed with solutions containing unlabeled products or NTC, indicating the aggregation of AuNPs after salt addition. Of note, our study exhibited a similar peak for solutions containing thiolated PCR products. However, the absorption spectra of solutions containing the unlabeled products or NTC were different from those previously reported [30,41]. Those studies showed a broad band ranging from 550 to 650 nm. This might be due to differing properties of the AuNPs used in each study. Nevertheless, the color change was obvious when the solutions were monitored by the naked eye.

Since it was demonstrated that the length of thiolated PCR products affects the degree of AuNP aggregation, increase in the length of PCR products leads to an enhancement of resistance to salt-induced aggregation [43]. In this study, 838 bp PCR products of *PCA3* were added to the detection system. The results revealed that this PCR length can protect the salt-induced aggregation. We also performed the test using 167 bp PCR products. However, this thiolated product did not block aggregation (data not shown). Importantly, our assay was highly specific for the *PCA3* target. The assay showed positive results (red color) when samples from prostate cancer cell lines were examined (Fig. 3B). In the literature, *PCA3* is significantly expressed in androgen receptor (AR)-positive PCa cells such as LNCaP and VCaP [44]. There is no false positive when tested with normal prostate cells (RWPE-1). Amplification of *GAPDH* conducted as a control validated the successful RNA isolation and cDNA synthesis from all cell lines (Fig. 3A). Gel electrophoresis also confirmed the specificity of the primers. Normally, AuNP colorimetric assays are moderately sensitive in the nanomolar range [45]. The detection limit of this developed assay was determined to be 31.25 ng which is in the same range as previous studies [32,41]. The results exhibited that different concentrations of PCR products formed a good linear relation with A520/A640 ratios (Fig. 4D). When the concentration of target *PCA3* was increased, the peak shape red-shifted (Fig. 4C). Furthermore, our assay provided higher sensitivity compared to gel electrophoresis (Fig. 4A and B). The developed method was able to detect PCR products with concentrations ≥31.25 ng which were not detected by gel electrophoresis. The colorimetric assay was rapid compared to gel-based detection, requiring < 10 min to complete.

To illustrate the potential clinical utility of this colorimetric assay, 15 urinary samples were assayed; good agreement was seen between our colorimetric assay results and qRT-PCR results (Table 2). As compared with qRT-PCR, the proposed assay cannot quantitate the copy or expression level of *PCA3* but is appropriate for use as a screening assay. The assay could visually detect *PCA3* in urine of all PCa patients, with different results observed when urine from both healthy controls and BPH patients were assayed (Figs. 5B, 6B, 7B). These results indicated that our assay can distinguish PCa patients from other participants. Moreover, our method also showed a good reproducibility over

independent runs (triplicate). Interestingly, some samples of PCa patients showed two bands of PCR products (838 and 611 bp) detected by gel electrophoresis. Blast analysis suggested that a band of 611 bp could be possibly due to the different transcript variant or isoform of *PCA3*. However, this phenomenon did not affect detection. Taken together, these results indicated that the developed colorimetric assay was applicable for detecting *PCA3* in urine and could be readily adapted for either the naked eye or spectrophotometrical readout platforms. Considering the sensitivity as well as speed and simplicity of naked eye detection of color change, this assay may provide a rapid and convenient preliminary screening tool for routine PCa diagnosis. However, further evaluation with larger sample sizes must be performed to verify the reliability of the assay.

5. Conclusions

In this study, a sensitive and specific AuNP-based colorimetric method for visual detection of *PCA3* in prostate cancer was successfully developed. This new method was based on interactions between thiolated PCR products and unmodified AuNPs. The positive and negative results were clearly distinguished by the naked eye, being red and blue color, respectively. Although it still required a RT-PCR step, post-PCR analysis with gel electrophoresis was not needed in this method. The incubation time was short and results were obtained within 10 min of RT-PCR completion. Moreover, a large number of samples could be tested simultaneously in 96-well microtiter plates. In short, this method was simple, rapid, cost-effective and did not require complicated instruments. Given these advantages, this assay has the potential to be used for *PCA3* detection in urine. Significantly, the proposed method is promising in that it discriminated PCa patients from healthy subjects and BPH patients based on differing expression levels of *PCA3* in their urine sediments. To the best of our knowledge, this is the first approach utilizing AuNPs to detect *PCA3* for the accurate diagnosis of prostate cancer.

Conflict of interest

The authors declare that they have no financial or commercial conflicts of interest.

Acknowledgements

This work was supported by National Research Council of Thailand and Health Systems Research Institute (grant number HSRI 60-026). Khin Phyu Pyar Htoo was supported by the Mahidol-Norway Capacity Building Initiative for ASEAN Scholarship.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2017, CA Cancer J. Clin. 67 (2017) 7–30.
[2] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer 136 (2015) E359–E386.
[3] C. Bax, G. Taverna, L. Eusebio, S. Sironi, F. Grizzi, G. Guazzoni, L. Capelli, Innovative diagnostic methods for early prostate cancer detection through urine analysis: a review, Cancers (Basel). 10 (2018) E123.
[4] J.H. Hayes, M.J. Barry, Screening for prostate cancer with the prostate-specific antigen test: a review of current evidence, JAMA 311 (2014) 1143–1149.
[5] R.A. Smith, V. Cokkinides, H.J. Eyre, American Cancer Society, American Cancer Society guidelines for the early of cancer, CA Cancer J. Clin. 54 (2004) 41–52.
[6] E.A. Heijnsdijk, A. der Kinderen, E.M. Wever, G. Draisma, M.J. Roobol, H.J. de Koning, Overdetection, overtreatment and costs in prostate-specific antigen screening for prostate cancer, Br. J. Cancer 101 (2009) 1833–1838.
[7] G. Aslan, B. Irer, S. Cimen, Y. Goktay, I. Celebi, B. Tuna, K. Yorukoglu, The performance of abnormal digital rectal examination for the detection of prostate cancer at stratified prostate specific antigen levels, Open J. Urol. 1 (2011) 67–71.
[8] M.J. Roobol, Is prostate cancer screening bad or good? Summary of a debate at the innovation in urology meeting, September 17–19, 2010, Milan, Italy, Eur. Urol. 59 (2011) 359–362.
[9] U.S. Preventive Services Task Force, D.C. Grossman, S.J. Curry, D.K. Owens, K. Bibbins-Domingo, A.B. Caughey, K.W. Davidson, C.A. Doubeni, M. Ebell, J.W.Jr. Epling, A.R. Kemper, A.H. Krist, M. Kubik, C.S. Landefeld, C.M. Mangione, M. Silverstein, M.A. Simon, A.L. Siu, C.W. Tseng, Screening for prostate cancer: US Preventive Services Task Force Recommendation Statement, JAMA 319 (2018) 1901–1913.
[10] M.J. Bussemakers, A. van Bokhoven, G.W. Verhaegh, F.P. Smit, H.F. Karthaus, J.A. Schalken, F.M. Debruyne, N. Ru, W.B. Isaacs, *DD3*: a new prostate-specific gene, highly overexpressed in prostate cancer, Cancer Res. 59 (1999) 5975–5979.
[11] D. Hessels, J.M. Klein Gunnewiek, I. van Oort, H.F. Karthaus, G.J. van Leenders, B. van Balken, L.A. Kiemeney, J.A. Witjes, J.A. Schalken, DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer, Eur. Urol. 44 (2003) 8–15.
[12] G.H. Leyten, D. Hessels, S.A. Jannink, F.P. Smit, H. de Jong, E.B. Cornel, T.M. de Reijke, H. Vergunst, P. Kil, B.C. Knipscheer, I.M. van Oort, P.F. Mulders, C.A. Hulsbergen-van de Kaa, J.A. Schalken, Prospective multicentre evaluation of *PCA3* and *TMPRSS2-ERG* gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer, Eur. Urol. 65 (2014) 534–542.
[13] H. Rittenhouse, A. Blase, B. Shamel, J. Schalken, J. Groskopf, The long and winding road to FDA approval of a novel prostate cancer test: our story, Clin. Chem. 59 (2013) 32–34.
[14] D.A. Sartori, D.W. Chan, Biomarkers in prostate cancer: what's new? Curr. Opin. Oncol. 26 (2014) 259–264.
[15] C.G. Ronnau, G.W. Verhaegh, M.V. Luna-Velez, J.A. Schalken, Noncoding RNAs as novel biomarkers in prostate cancer, Biomed. Res. Int. 2014 (2014) 591703.
[16] J. Groskopf, S.M. Aubin, I.L. Deras, A. Blase, S. Bodrug, C. Clark, S. Brentano, J. Mathis, J. Pham, T. Meyer, M. Cass, P. Hodge, M.L. Macairan, L.S. Marks, H. Rittenhouse, APTIMA *PCA3* molecular urine test: development of a method to aid in the diagnosis of prostate cancer, Clin. Chem. 52 (2006) 1089–1095.
[17] A. Nicholson, J. Mahon, A. Boland, S. Beale, K. Dwan, N. Fleeman, J. Hockenhull, Y. Dundar, The clinical effectiveness and cost-effectiveness of the PROGENSA prostate cancer antigen 3 assay and the Prostate Health Index in the diagnosis of prostate cancer: a systematic review and economic evaluation, Health Technol. Assess. 19 (2015) 1–191) i-xxxi.
[18] M.S. Han, A.K. Lytton-Jean, B.K. Oh, J. Heo, C.A. Mirkin, Colorimetric screening of DNA-binding molecules with gold nanoparticle probes, Angew. Chem. Int. Ed. Engl. 45 (2006) 1807–1810.
[19] J. Liu, Y. Lu, Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes, Nat. Protoc. 1 (2006) 246–252.
[20] L.H. Xiong, X. He, J. Xia, H. Ma, F. Yang, Q. Zhang, D. Huang, L. Chen, C. Wu, X. Zhang, Z. Zhao, C. Wan, R. Zhang, J. Cheng, Highly sensitive naked-eye assay for enterovirus 71 detection based on catalytic nanoparticle aggregation and immunomagnetic amplification, ACS Appl. Mater. Interfaces 9 (2017) 14691–14699.
[21] C.A. Mirkin, R.L. Letsinger, R.C. Mucic, J.J. Storhoff, A DNA-based method for rationally assembling nanoparticles into macroscopic materials, Nature 382 (1996) 607–609.
[22] J.J. Storhoff, A.A. Lazarides, R.C. Mucic, C.A. Mirkin, R.L. Letsinger, G.C. Schatz, What controls the optical properties of DNA-linked gold nanoparticle assemblies? J. Am. Chem. Soc. 122 (2000) 4640–4650.
[23] K. Saha, S.S. Agasti, C. Kim, X. Li, V.M. Rotello, Gold nanoparticles in chemical and biological sensing, Chem. Rev. 112 (2012) 2739–2779.
[24] P. Tiet, K.C. Clark, J.O. McNamara, J.M. Berlin, Colorimetric detection of *Staphylococcus aureus* contaminated solutions without purification, Bioconjug. Chem. 28 (2017) 183–193.
[25] J.H. Lee, Z.D. Wang, J.W. Liu, Y. Lu, Highly sensitive and selective colorimetric sensors for Uranyl (UO22+): development and comparison of labeled and label-free DNAzyme-gold nanoparticle systems, J. Am. Chem. Soc. 130 (2008) 14217–14226.
[26] C.M. Niemeyer, U. Simon, DNA-based assembly of metal nanoparticles, Eur. J. Inorg. Chem. (2005) 3641–3655.
[27] D. Prasad, A.S. Vidyarthi Shankaracharya, Gold nanoparticles-based colorimetric assay for rapid detection of *Salmonella* species in food samples, World J. Microbiol. Biotechnol. 27 (2011) 2227–2230.
[28] Z.M. Liu, J.C. Zhu, C.Y. Yang, X.H. Li, Visual detection of *Listeria monocytogenes* using unmodified gold nanoparticles based on a novel marker, Anal. Methods-Uk. 7 (2015) 8159–8164.
[29] Y.L. Jung, C. Jung, H. Parab, T. Li, H.G. Park, Direct colorimetric diagnosis of pathogen infections by utilizing thiol-labeled PCR primers and unmodified gold nanoparticles, Biosens. Bioelectron. 25 (2010) 1941–1946.
[30] Z. Liu, X. Xia, C. Yang, L. Wang, Visual detection of Maize chlorotic mottlevirus using unmodified gold nanoparticles, RSC Adv. 5 (2015) 100891–100897.
[31] H. Han, W. Yi, D. Hou, T. Huang, Z. Hao, AuNPs-based colorimetric assay for identification of chicken tissues in meat and meat products, J. Nanomater. 16 (2015) 276.
[32] M.S. Verma, J.L. Rogowski, L. Jones, F.X. Gu, Colorimetric biosensing of pathogens using gold nanoparticles, Biotechnol. Adv. 33 ((2015) 666–680.
[33] M.M. Hussain, T.M. Samir, H.M. Azzazy, Unmodified gold nanoparticles for direct and rapid detection of Mycobacterium tuberculosis complex, Clin. Biochem. 46 (2013) 633–637.
[34] S. Sharma, P. Mandal, T. Sadhukhan, R. Roy Chowdhury, N. Ranjan Mondal, B. Chakravarty, T. Chatterjee, S. Roy, S. Sengupta, Bridging links between long noncoding RNA HOTAIR and HPV oncoprotein E7 in cervical cancer pathogenesis, Sci. Rep. 5 (2015) 11724.
[35] J.B. de Kok, G.W. Verhaegh, R.W. Roelofs, D. Hessels, L.A. Kiemeney, T.W. Aalders, DD3(PCA3), a very sensitive and specific marker to detect prostate tumors, Cancer Res. 62 (2002) 2695–2698.
[36] M. Shen, W. Chen, K. Yu, Z. Chen, W. Zhou, X. Lin, Z. Weng, C. Li, X. Wu, Z. Tao, The diagnostic value of *PCA3* gene-based analysis of urine sediments after digital rectal examination for prostate cancer in a Chinese population, Exp. Mol. Pathol. 90

(2011) 97–100.
[37] H. Moradi Sardareh, M.T. Goodarzi, R. Yadegar-Azari, J. Poorolajal, S.H. Mousavi-Bahar, M. Saidijam, Prostate cancer antigen 3 gene expression in peripheral blood and urine sediments from prostate cancer and benign prostatic hyperplasia patients versus healthy individuals, Urol. J. 11 (2014) 1952–1958.
[38] M. Li, D. Zhou, W. Zhang, S. Gao, X. Zhou, Urine *PCA3* mRNA level in diagnostic of prostate cancer, J. Cancer Res. Ther. 14 (2018) 864–866.
[39] M. Schmid, J. Hansen, F.K. Chun, Urinary prostate cancer antigen 3 as a tumour marker: biochemical and clinical aspects, Adv. Exp. Med. Biol. 867 (2015) 277–289.
[40] A. de la Taille, Progensa *PCA3* test for prostate cancer detection, Expert. Rev. Mol. Diagn. 7 (2007) 491–497.
[41] Z. Fu, X. Zhou, D. Xing, Rapid colorimetric gene-sensing of food pathogenic bacteria using biomodification-free gold nanoparticle, Sens. Actuators B Chem. 182 (2013) 633–641.
[42] W.R. Glomm, Functionalized gold nanoparticles for applications in bionano-technology, J. Dispers. Sci. Technol. 26 (2005) 389–414.
[43] H.W. Walker, S.B. Grant, Coagulation and stabilization of colloidal particles by adsorbed DNA block copolymers: the role of polymer conformation, Langmuir 12 (1996) 3151–3156.
[44] E. Ozgur, A.I. Celik, E. Darendeliler, U. Gezer, *PCA3* silencing sensitizes prostate cancer cells to enzalutamide-mediated androgen receptor blockade, Anticancer Res. 37 (2017) 3631–3637.
[45] R. Kanjanawarut, X. Su, Colorimetric detection of DNA using unmodified metallic nanoparticles and peptide nuclei acid probes, Anal. Chem. 81 (2009) 6122–6129.